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# Histological Investigations on the Tyrolean Ice Man

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*ABSTRACT* The 5,200-year-old Tyrolean Ice Man discovered in 1991 in the Ötztal Alps is the world's most ancient known human glacier mummy. Histological investigation was aimed at 1) optimizing specimen preparation and 2) documenting the preservation state of (sub)cellular components. Minute pieces of frozen tissue were removed endoscopically from rib bone and cartilage, major blood vessels, oral cavity and alimentary tract, liver, spleen, diaphragm, respiratory system, femoral muscle and nerve, sympathetic trunk, brain, and skin. Double fixation with glutaraldehyde followed by osmium tetroxide and embedding in Epon/Araldite epoxy resins proved to be the method of choice for both light and transmission electron microscopy combined with classical histochemistry. In particular, mild evacuation of the desiccated tissue was determined to be essential to ensure homogeneous infiltration with fixatives and resins; as a result, sections of excellent quality could be obtained with any kind of sample. With regard to the preservation degree of (sub)cellular components, distinct tissue-specific patterns were observed. There were highly intact skeletal and connective tissues proper, however, most interestingly, there were remarkably intact nervous tissue components as well. By contrast, epithelial, muscle, and reticular connective tissues as well as blood had generally disintegrated due to autolysis, freeze/thaw damage, and adipocere formation. For a tentative interpretation of these patterns, we considered general aspects of cryopreservation, such as physicochemical properties of subcellular constituents and tissue physiology. Am J Phys Anthropol 106:521–532, 1998. 

In 1991, an exceptionally well-preserved, prehistoric male corpse was discovered in a glacial field in the Ötztal Alps at 3,200 meters above sea level near the Italian-Austrian border. For details on the recovery, see Seidler et al. (1992). The frozen body proved to be almost completely mummified, presumably as a result of processes akin to freeze drying (Ambach et al., 1992). Based on accelerator mass spectrometry and conventional archaeological dating, its absolute age was estimated to be approximately 5,200 years, i.e., an individual from the Late Neolithic Age (Bonani et al., 1992). Hence, the Tyrolean Ice Man, also known as the "Man from Hauslabjoch," "Similaun Man", or "Ötzi," is the most ancient human glacier mummy discovered to date.

The aims of the histological investigations on the Tyrolean Ice Man were 1) to optimize specimen preparation according to the tissues' specificities, and 2) to document the preservation state of (sub)cellular compo-

Dedicated to Prof. Werner Platzer (Head and Chairman of the Institute of Anatomy, Medical Faculty, University of Innsbruck) on the occasion of his retirement.

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nents. We employed chemical fixation followed by resin embedding or cryomicrotomy and classical histochemistry for light microscopy (LM) and transmission electron microscopy (TEM). When our data are compared with the literature on other mummies, the very particular conditions of preservation in each case must be considered. For instance, permafrost mummies, such as 15th century Greenland mummies (Hart-Hansen et al., 1991) and pre-Columbian Andean mummies from El Plomo or Nevado Amapato (Reinhard, 1996), may compare well with the Ice Man (for review, see Zimmermann, 1996). By contrast, there is less congruence with finds from the deserts of Taklimakan or Northern Chile or with bog bodies from Northern Europe (for review, see Spindler et al., 1996). In these cases, decomposition was impeded by exposure to salt, dry sand, or tannic acids, respectively; analogous mechanisms of preservation and, thus, restricted comparability hold true for artificially embalmed mummies.

In the present contribution, we show representative data on a broad range of tissue samples obtained from the Tyrolean Ice Man. The distinct preservation patterns that were observed proved to be highly specific. We therefore attempt to interpret some of them by considering general aspects of cryopreservation (e.g., physicochemical properties of subcellular constituents and tissue physiology).

Results of this study were presented in part in abstract form (Hess et al., 1996a,b).

## MATERIALS AND METHODS

### Sampling

Minute quantities of tissue were removed endoscopically under sterile conditions by employing titanium tools, as described in detail by Gaber et al. (1995), Gunkel et al. (1997), and Platzer et al. (1998). The total weight of samples taken for biological and medical analyses was approximately 10 g, and the mummy's weight was 13.29 kg (Künzel, unpublished observations).

According to topographic and macroscopic criteria, samples for histological investigation were obtained from rib bone and cartilage, vena cava, thoracic aorta, oral cavity, stomach, small and large intestine, liver, spleen, diaphragm, nasal cavity, larynx, lung [see also the analytical electron microscopic work by Pabst and Hofer (1998) on the Ice Man's lung deposits], femoral muscle, femoral nerve, sympathetic trunk, cerebral cortex, and skin (in total, more than 50 tissue samples). Control samples were obtained from a present-day human glacier corpse (i.e., a mountain climber who had been lost in the 1920s in the Ötztal Alps and released from the glacier approximately 70 years later).

### Fixation

Immediately after sampling, the more or less frozen tissue pieces were dissected into small cubes 1–4 mm side length. Subsequently, the specimens were immersed in cold fixative (4% weight/volume formaldehyde and/or 2.5% volume/volume glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, at 4°C) and kept for approximately 90 seconds under mild vacuum to remove air from the leather-like tissue (evacuation by hand within a syringe). This procedure is common for botanical specimens, facilitating homogeneous infiltration with fixatives and embedding media (Hayat, 1989). After aldehyde fixation (12–24 hours at 4–25°C), the specimens were optionally postfixed with 1% (weight/volume) aqueous osmium-tetroxide ($OsO_4$; 8 hours at 4°C) followed by ethanol and/or acetone dehydration.

### Embedding and microtomy

Pilot experiments with paraffin embedding produced unsatisfactory results in terms of sectioning properties and identification of structural details as well as poor or uncertain reliability of staining specificity; thus, we generally used resin embedding. Specimens were infiltrated with resin monomers at room temperature for 3 days with continual motion. Epoxy or acrylic resins were heat cured (Epon, Araldite, or Unicryl: British Biocell International, Cardiff, United Kingdom; LR white: London Resin Company, London, United Kingdom). Resin blocks were trimmed with a motor-driven abrasive wheel (Reichert Ultratrim, Leica; Vienna, Austria). Semithin sections (0.2–0.5 µm) or ultrathin sections (80–120 nm) were cut from block faces of 4 mm or 2 mm side

length, respectively, by using an ultramicrotome (Reichert Ultracut-S, Leica) equipped with diamond knives (Diatome, Biel, Switzerland) at a cutting speed of 0.4–1.0 mm per second (note: cross links between mummified tissues and the embedding resins are only weak; to prevent tissue components from breaking off the resin, it proved essential to avoid trimming by hand with razor blades or cutting sections thicker than 0.5 µm). Semithin sections were transferred with a wire loop onto glass slides; in the case of fragile embeddings, sections were allowed to attach onto the glass slide merely by drying at room temperature followed by heating the glass to approximately 90°C. Hence, the fragmentation and/or floating away of delicate sections prior to or during staining was completely avoided. Ultrathin sections were collected onto uncoated or Formvar carbon-coated copper or gold 200-mesh thin bar grids.

### Cryomicrotomy

0.5–2.5 µm semithin cryosections (Tokuyasu, 1973) were cut with glass knives at from -70°C to -90°C by using a Reichert Ultracut-S ultramicrotome equipped with a Reichert FC-S cryochamber.

### Staining and microscopy

***Semithin sections.*** Staining was performed as follows (see also, Pearse, 1980): 1) Toluidine blue; 2) Toluidine blue followed by acid fuchsin (see pp. 326–327 in Hayat, 1989); 3) Victoria blue B for elastic material (see Snodgress et al., 1972) applied to deplastinized epoxy sections (Hayat, 1989); 4) enzymic digestion of elastin with elastase type III (Montes et al., 1985) applied to deplastinized and optionally oxidized epoxy sections followed by general or elastin-specific staining; 5) Coomassie brilliant blue for proteins (Fisher, 1968) applied to epoxy sections or cryosections; 6) Sudan black B for lipid localization on cryosections; and 7) 0.1 µg/ml diamidino-2-phenylindole (DAPI; Manzini et al., 1983) for DNA localization on cryosections. The sections were viewed with a Photomikroskop III or a Axiophot (Zeiss, Oberkochen, Germany).

***Ultrathin sections.*** The following procedures were used for processing of ultrathin sections: 1) overall staining with 0.5% weight/volume aqueous uranyl acetate and alkaline lead citrate (Reynolds, 1963); 2) periodic acid-thiocarbohydrazide-silver proteinate stained polysaccharides with 1–2 glycol groups (Thiéry, 1967; Courtoy and Simar, 1974; Erdos, 1986), as described previously (Hess and Hesse, 1994); 3) thiocarbohydrazide-silver proteinate staining, which indicated unsaturated lipids (Rowley and Dahl, 1977), as described previously (Hess and Hesse, 1994). Sections were examined with an electron microscope at 60, 80, or 100 kV (EM 10A, Zeiss; CM120 Philips, Eindhoven, Netherlands).

Note that, for reasons of brevity and legibility, we usually used the term "cells" instead of "remnants/residues/remains of cells" in the following text.

## RESULTS AND DISCUSSION

### General methodological remark

For conservational reasons, the removal of tissue samples from this unique corpse was limited to the absolutely indispensable minimum. This curatorial decision was supported by the very first histological analysis of the digestive tract and liver, in which poor preservation was observed. Considering the lack of preservation of visceral tissues, together with the minute size of the specimens, our approach was not able to reveal any possible pathological changes.

### Technical notes

Double fixation with aldehydes and osmium-tetroxide followed by Epon/Araldite embedding proved to be the preparation method of choice for LM and TEM analysis. Excellent, reproducible results were obtained with any kind of tissue. Special procedures to soften desiccated tissue, as introduced by Ruffer (1921) or Sandison (1955), for instance, were not necessary. However, sufficient evacuation of samples prior to fixation combined with prolonged resin infiltration was found to be essential in order to obtain homogeneous blocks and, in turn, coherent sections. No further improvement was achieved by embedding in acrylic resins

instead of Epon/Araldite, which is consistent with a remark by Williams et al. (1995) on skin samples from the Ice Man. Spurr's low-viscosity epoxy resin mixture (Spurr, 1969) was not tested because of its potential extractive properties (see Hess, 1990).

Due to the specimens' properties, folds in semithin and ultrathin sections were inherent in all preparations.

## Histological observations

Due to the mummy's low actual weight (approximately 13 kg), as expected, the soft tissues displayed signs of considerable shrinkage, e.g., extremely dense arrangement of collagen fibrils. A certain rehydration of the mummified tissues, however, was achieved by exposing the specimens overnight to aqueous fixatives.

Concerning the preservation state of (sub)-cellular constituents, it has to be emphasized that the patterns observed throughout all samples analyzed are highly regular. Furthermore, the degree of preservation appears to be specific for the various types of tissues/cells/subcellular constituents.

***Skeletal and connective tissues proper (e.g., compact bone, hyaline cartilage, elastic arteries, large veins).*** The gross architecture of rib bone (Figs. 1, 2) and cartilage as well as of major blood vessels, such as thoracic aorta (Fig. 7) or vena cava, is easily recognized (for details, see figure legends; note: reticular connective tissues are discussed below).

Extracellular components, in particular collagen and elastin, are well preserved and could always be identified. Collagen fibrils still display specific ultrastructural features, e.g., approximately 64/67 nm periodicity (Fig. 4), and birefringence under polarized light. Elastic material appeared completely amorphous, and no peripheral microfibrils were seen (Fig. 8). Its chemical nature was assessed histochemically. Commonly used dyes, such as orcein or resorcin-fuchsin, had proven to be nonspecific in pilot experiments with paraffin and deplastinized resin sections, a feature already known from other ancient tissues (Sandison, 1963). Victoria blue B as well as sequential staining with Toluidine blue and acid fuchsin (Hayat, 1989) gave reliable results, as verified by elastase digestion according to Montes et al. (1985). Hayat's recipe proved to be particularly apt for making a clear distinction between elastic material and collagen on black-and-white photomicrographs (Fig. 7).

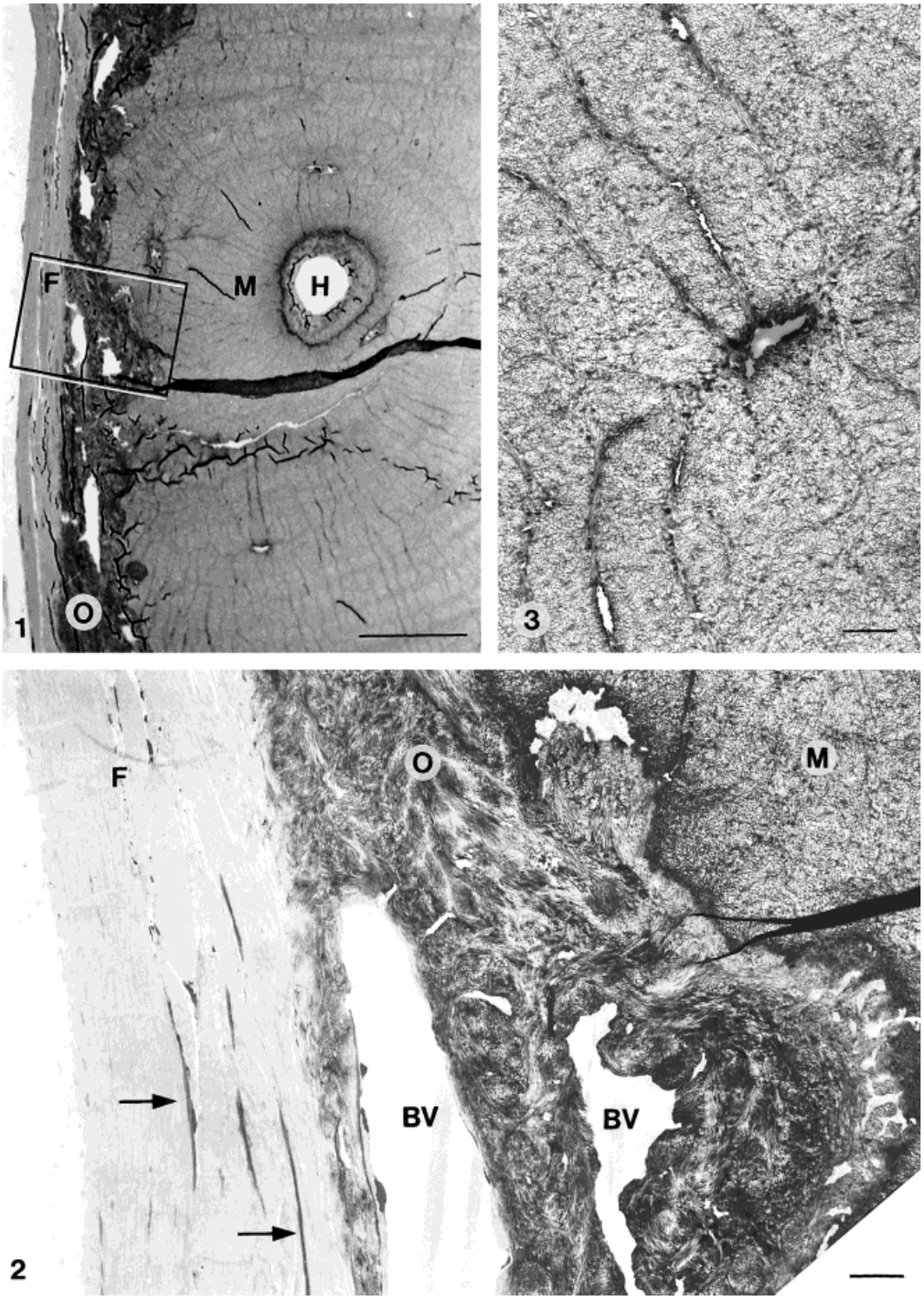
With regard to the cells of these connective tissues, we observed generally well-defined, spindle-shaped structures with amorphous contents, presumably fibrocytes, located between densely packed collagen fibrils (Figs. 2, 4); thus, similar elongate structures occurring within the perineurium of the femoral nerve likely represent perineural cells. Osteocytes are no longer visible, except for their outlines (Fig. 3). Chondrocytes, which were identified clearly in rib cartilage, consistently showed distinct subcellular compartments resembling nuclei (Fig. 5). However, the internal organization of cytoplasm and nucleoplasm had not been preserved. Moderate, although distinct, DAPI fluorescence observed within the chondrocytes strongly indicated DNA, possibly nuclear DNA. (Fig. 6; note that Handt et al., 1994, studied material from the same rib sample.)

***Nervous tissue (e.g., peripheral nerves, vegetative ganglia, brain).*** Tissue samples obtained from the Ice Man's femoral nerve and sympathetic trunk regularly displayed the concentric membrane lamellae of myelin sheaths (Figs. 9, 11, 12) embedded within collagenous endoneurium and surrounded by flattened perineural cells. Myelinated nerve fibers of the diaphragm, skin (Fig. 10), and cerebral cortex also proved to be preserved. Note, however, that some

Fig. 1. Cross section through an osteon from rib bone with adjacent periosteum. F, stratum fibrosum of periosteum; H, Haversian canal; M, mineralized bone matrix; O, stratum osteogenicum of periosteum. The window frames the corresponding area shown in Figure 2. Light microscopy (LM), Toluidine blue staining (Tol). Scale bar = 25 µm.

Fig. 2. Rib bone periosteum: fibrocytes (arrows) within the stratum fibrosum and blood vessels (BV) within the stratum osteogenicum can be seen. Transmission electron microscopy (TEM), uranyl acetate and lead staining (UA/Pb). Scale bar = 2.5 µm.

Fig. 3. Rib bone showing a ghost of an osteocyte (tangential section) with clearly visible canaliculi. TEM; (UA/Pb). Scale bar = 2.5 µm.




Figs. 1–3.

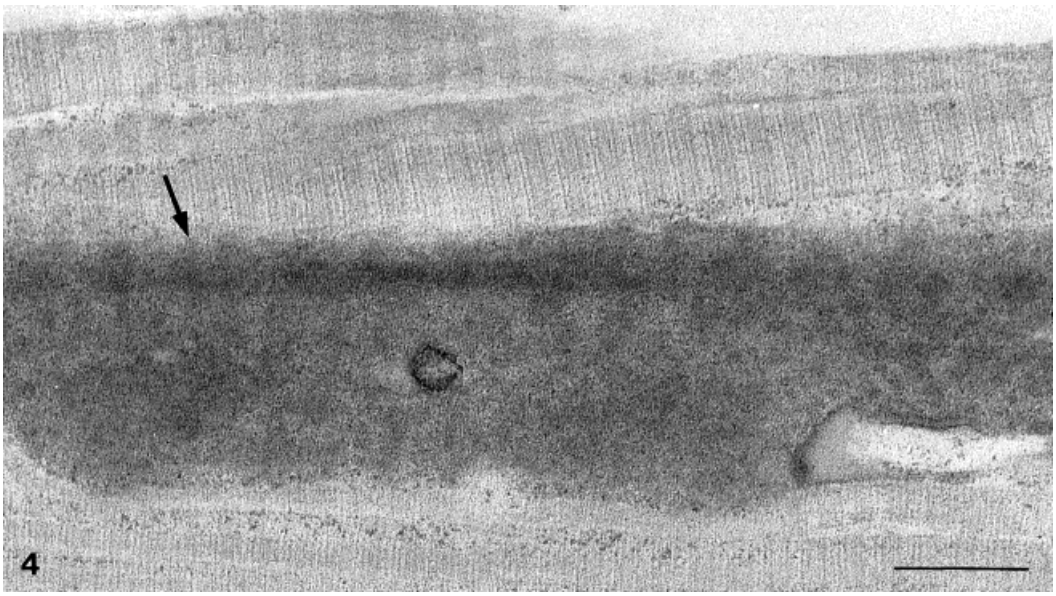
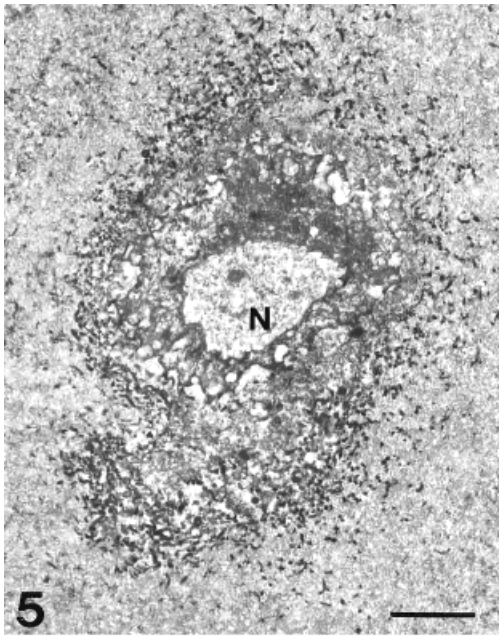



Fig. 4. Collagen fibres with typical periodicity of approx. 64 nm (stratum fibrosum of rib bone periosteum). The arrow indicates a fibrocyte. TEM; UA/Pb. Scale bar = 0.2 µm.

nerve fiber remnants showed completely disordered ultrastructure. We assume that repeated freezing and thawing of the Ice Man during the preceding 5,000 years (including the recovery and subsequent storage of the corpse at -6°C) accounted for local structural breakdown of myelin sheaths and rearrangement ("recrystallization") of myelin constituents (for details on the latter phenomenon, see, e.g., Erk et al., 1996). Patterns that resembled somewhat the nodes of Ranvier were observed occasionally, Schmidt-Lanterman clefts could not be identified at all. Stains for lipid and protein (Sudan black and Coomassie blue, respectively) applied to semithin cryosections and/or resin sections reacted positively with the myelin remains (see, e.g., Fig. 13); at the electron microscopic level, unsaturated lipids and periodic acid-Schiff-reactive carbohydrates could be localized (see, e.g., Fig. 12). These photomicrographs show for the first time, to our knowledge, subcellular components of nervous tissue preserved over millennia in a naturally mummified human (see Doran et al., 1986; Gerszten and Martinez, 1995). Photomicrographs showing recognizable structural patterns of mummified myelin have only been published from modern (approximately 50-year-old) samples (Radanov et al., 1992); Aichel (1927) briefly mentioned recognizable myelin sheaths in an approximately 1,500-year-old bog corpse but did not provide drawings or photographs; Gouzhang et al. (1979) published micrographs from an approximately 2,000-year-old female corpse showing remains of peripheral nerve fibers, but stated that ". . . details of their submicroscopic structures could not be distinguished." The nerve cell bodies observed within a ganglion from the sympathetic trunk (Fig. 11) or the cerebral cortex no longer showed internal structures, nor did the residues of axoplasm (Fig. 10) or Schwann cells' cytoplasm that were occasionally identified as well.

***Epithelial tissue (e.g., in digestive and respiratory tract; glands, endothelia), muscle tissue (e.g., smooth muscle of blood vessels and digestive tract, skeletal muscle), reticular connective tissue (e.g., spleen, bone marrow), and blood.*** Epithelial and reticular connective tissues as well as blood cells generally suffered from conspicuous disintegration. They were trans-



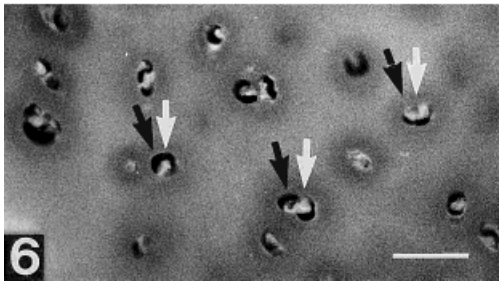
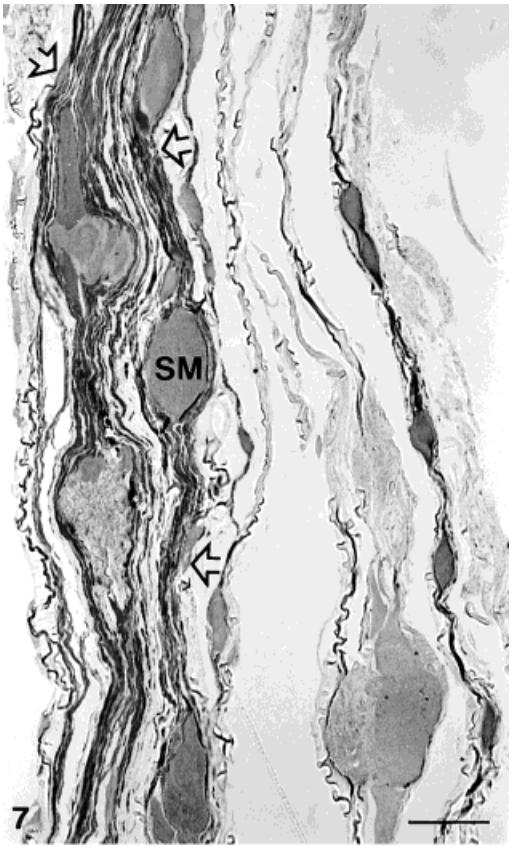




Fig. 5. Chondrocyte (from rib cartilage) with an internal compartment resembling the nucleus (N). TEM; UA/Pb. Scale bar = 2 µm.

Fig. 6. Diamidino-2-phenylindole (DAPI) fluorescence (arrows) indicating DNA preservation in the chondrocytes of rib cartilage (2.5-µm cryosection). LM. Scale bar = 50 µm.

formed into masses of amorphous to crystalline material, so-called "white grave wax" or adipocere (see also Bereuter et al., 1996a,b; Mayer et al., 1997). In particular, this held true for the entire mucosa of the stomach,



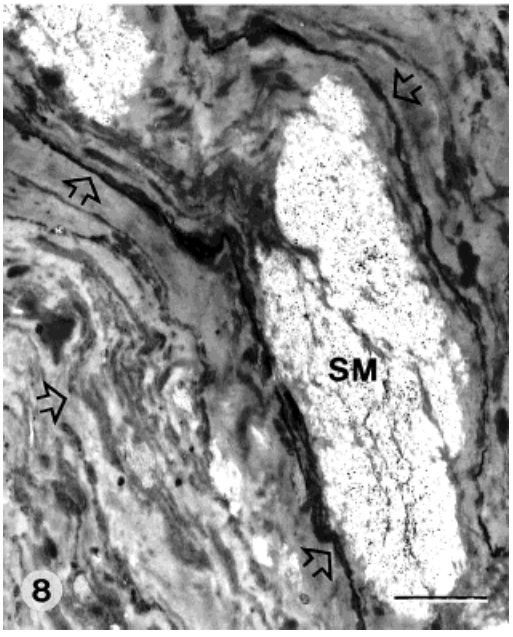




Fig. 7. Details of the thoracic aorta. Darkly stained elastic material (open arrows) is well set off against the moderately stained collagen fibrils. SM, putative remnants of smooth muscle cells. LM, Toluidine blue/acid fuchsin. Scale bar = 10 µm.

Fig. 8. Details of the same thoracic aorta shown in Figure 7. TEM; UA/Pb. Scale bar = 2 µm.

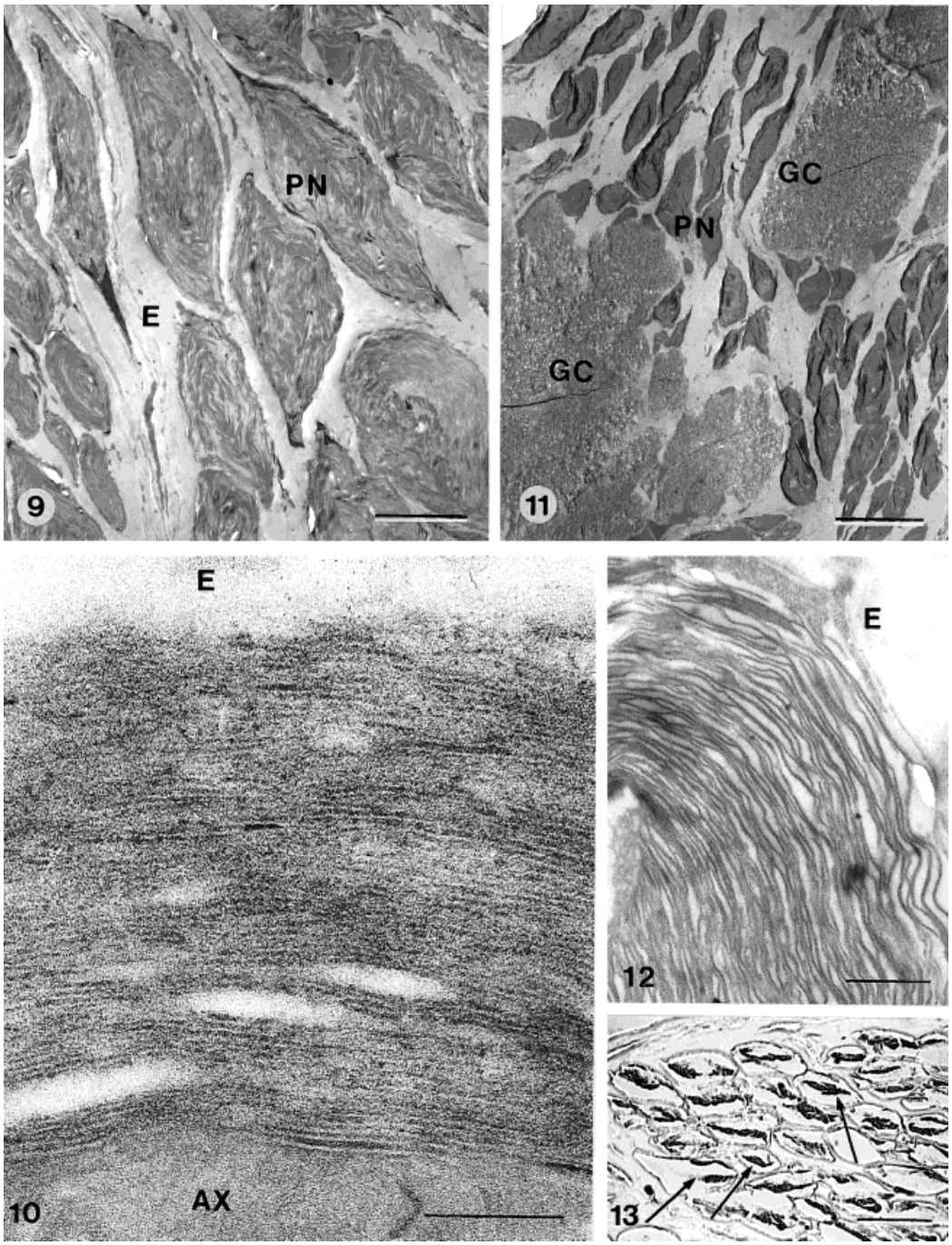


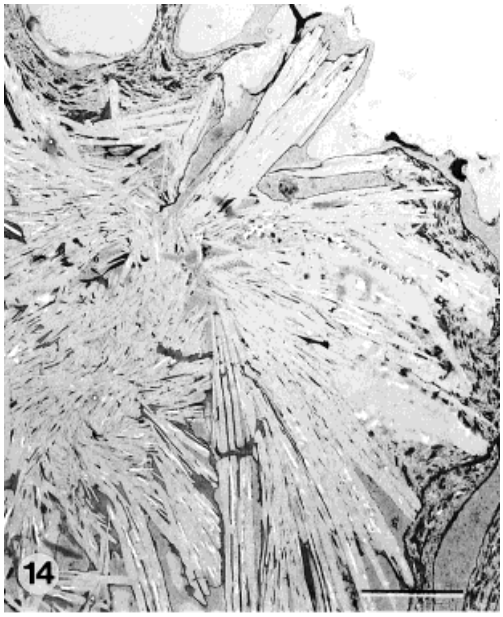

Fig. 9. Peripheral nerve fibres (PN) embedded within collagenous endoneurium (E; detail of femoral nerve in cross section). TEM. Scale bar = 5 µm.

Fig. 10. The multilamellar patterns of a peripheral nerve's myelin sheath (from skin). Axon remains (AX) appear amorphous. TEM, UA/Pb. Scale bar = 0.1 µm.

Fig. 11. Vegetative ganglion with thin nerve fibres and disintegrated ganglion cells (GC). TEM. Scale bar = 5 µm.

Fig. 12. Ultrastructural localization of periodic acid-Schiff-reactive carbohydrates in femoral nerve myelin. TEM; periodic acid-thiocarbohydrazide-silver proteinate staining. E, endoneurium. Scale bar = 0.5 µm.

Fig. 13. Sudan black staining of myelin lipid (arrows) in a 1-µm cryosection through the femoral nerve. LM. Scale bar = 20 µm.



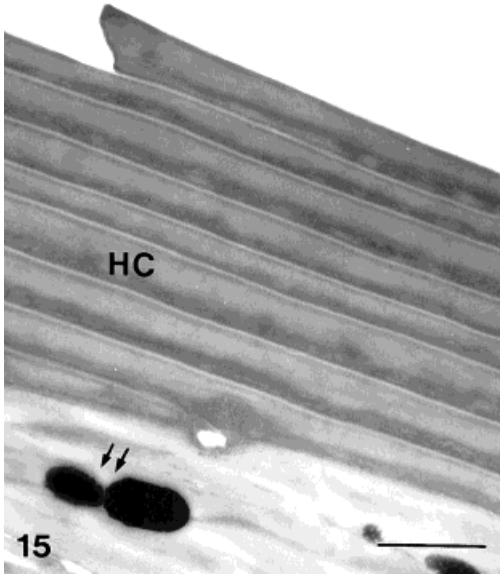




Fig. 14. Crystalline adipocere observed in liver samples. TEM; UA/Pb. Scale bar = 5 µm.

Fig. 15. Inner hair cuticle (HC) with preserved cellular patterns. Double arrow marks a pigment granule. TEM; UA/Pb. Scale bar = 1 µm.

the small and large intestine, as well as the lung, and, likewise, for the liver parenchyma (Fig. 14), peripheral blood, bone marrow, and the splenic pulp. These findings contrast in part with reports of remarkably well-preserved blood and epidermal cells in artificial and natural dry mummies (see, e.g., Lewin, 1967; Zimmerman, 1973; Riddle et al., 1975; Perrin et al., 1994; Gerszten et al., 1997). The only examples of recognizable epithelial remains in the Ice Man are the clearly defined cellular patterns of the hair cuticle (Fig. 15).

With regard to muscle tissue, the outlines of cells/fibers sometimes appeared still discernible, but the cells' internal architecture was generally destroyed (see, e.g., Figs. 7, 8). It is of note that Zimmerman and Tedford (1976) could show cross striations preserved in skeletal muscle from a 21,000-year-old mammoth.

Control samples of any kind of soft tissue obtained from a present-day glacier corpse showed almost complete structural breakdown as a consequence of adipocere formation.

## Hypotheses dealing with the tissue-specific preservation patterns of the Ice Man's tissues

It seems reasonable to characterize briefly the major mechanisms that account generally for tissue disintegration in glacier-corpses. In chronological order, these are 1) autolysis and bacterial activity (biological destruction; gram-negative bacteria were observed in the digestive tract and within the lumen of a Haversian canal of rib bone); 2) freeze/thaw-damage, i.e., (intra)cellular ice crystallization, which causes membrane disruption and, in turn, confluence of (sub)cellular compartments (physical destruction); and 3) transformation of molecules into adipocere (chemical destruction; note: the mechanisms that account for this phenomenon, which has been known for centuries, are still enigmatic in detail; for reviews, see Bereuter et al., 1996a; Mayer et al., 1997). These destructive forces act in a cumulative way (in this context, see also Andreasen et al., 1991).

In the following, we consider some properties of (sub)cellular constituents that presumably contributed to impede disintegration of the Ice Man's body. First, structures that are distant from sites of enzymic activity were clearly not affected by autolysis,

e.g., myelin sheaths and extracellular fibers. Low metabolic activity, too, may have limited autolysis, for instance, in chondrocytes. Second, freeze/thaw damage was limited locally by the chemical composition of subcellular constituents: that is, high proportions of lipids and/or low water content of the myelin sheaths and dense collagenous tissue, respectively. Analogously, the specific chemical composition of cytoplasm and nucleus (i.e., different freezing properties) may account for the preservation of the nuclear compartment as a distinct structural entity in chondrocytes. Third, one should mention the physical properties of subcellular components: Highly ordered structures, such as myelin (being considered as "paracrystalline": Fernández-Morán, 1962) and collagen fibrils, essentially withstood destruction resulting from crystallization processes such as ice crystallization and adipocere formations. Finally, the isolated location of some kinds of cells should also be considered. The template of extracellular matrix and fibers, within which fibrocytes, perineural cells, and chondrocytes are embedded, may have helped to retain their more or less disintegrated residues in place, that is, to preserve at least the individual contours of these cells.

On the other hand, metabolically active cells that are rich in protein and/or water, such as internal epithelia (e.g., glands), the nerve cell bodies, reticular connective tissues, blood, and muscle, as well as the digestive tract with its bacteria, generally did not withstand structural disintegration and/or transformation processes (i.e., autolysis, bacterial decomposition, freeze/thaw damage, adipocere formation). Together, our histological data, in general, are consistent with reports on the molecular state of the Ice Man's tissues (i.e., partially well-preserved lipoidal components: Williams et al., 1995; Makristathis et al., 1996; more or less degraded proteins: Lubec et al., 1994; and more or less degraded nucleic acids: Handt et al., 1994).

## CONCLUSIONS AND OUTLOOK

Slight modifications of standard preparation procedures for electron microscopy allowed detailed microscopic analysis of a broad range of tissue samples obtained from the Tyrolean Ice Man. According to ultrastructural criteria, this naturally mummified, prehistoric human, in part, remained remarkably well preserved over millennia. Whether tissue components still exhibit lectin-binding or immunoreactivity (see Fulcheri, 1995) is currently under investigation. Apart from the morphological findings, methodological notes may be of interest for others who are investigating similar finds.

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